Chapter 16
Contribution of Proteomics Research to Understanding *Botrytis* Biology and Pathogenicity

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Abstract Recent work has clearly shown the capacity of proteomics-based methodologies to establish the roles played by specific proteins in different biological processes. Beyond the study of genes, it has been established that proteins are the relevant set to be analyzed in research aiming to solve specific biological questions. Proteomics approaches can be categorized according to three different methodologies; gel-based, mainly two-dimension gel electrophoresis (2-DE); gel free, based on liquid chromatography-mass spectrometry (LC-MS); and quantitative proteomics, by isobaric markers. Most of these methodologies have been applied to studies of the proteome of *Botrytis cinerea*. Since the publication of the first proteomics report on *Botrytis*, technological advances have accelerated the identification of global protein content. Clearly, the publication of the *B. cinerea* genome has been of tremendous value to the proteomics research community; this has supported the accurate identification, through MS, of this fungus' peptides. This landmark event has greatly facilitated the development of proteomics studies exploring the biology of the fungus; to date, mainly mycelium samples have been used. Only a few reports have aimed at the study of fractions of the total proteome, and all of these are focused on the secretome. The role of several particular proteins related to fungal pathogenicity, metabolism, biology, etc. has been elucidated, but the number of *Botrytis* proteins found, as a proportion of the total proteins predicted from the genome, remains below 10 %. There is much work to be done.

Keywords Subproteome • Secretome • 2-DE • LC-MS • Shotgun proteomics

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16.1 Introduction

For most of the last 30 years the study of phytopathogenic fungi has been undertaken from a classical viewpoint, through the use of molecular biology techniques based on the isolation and characterization of individual genes and proteins, one by one. In the late 1990s and early 2000s, the development of large-scale DNA sequencing technology produced a significant breakthrough, allowing the publication of many fungal genomic sequences. After the publication of the first fungal genome in 1996, (Dujon 1996), the development of functional genomic analyses and large-scale techniques such as transcriptomics, proteomics and, more recently, metabolomics, effectively transformed research into plant fungal pathology. This evolution, from the study of genomics to the application of these new research techniques, has led some authors to describe the current period as the "post-genomic era" (Fernandez-Acero et al. 2007a; James 1997; Kim et al. 2007). Currently, among the possibilities that "-omics" offer towards achieving a better understanding of *Botrytis cinerea* biology, high-throughput proteomics techniques, in conjunction with advances in bioinformatics, have been shown to be very useful and effective tools in the research of various aspects related to biological, metabolic, physiological and pathological processes, as we will describe in the course of this chapter.

Proteomics is the science concerned with the study of the structure, function and interaction of the whole set of proteins codified by an organism (Wilkins et al. 1995). It comprises a wide range of tools, techniques and technological approaches for the systematic, large-scale analysis of the proteome; this latter term has, in turn, been defined as the complete set of proteins expressed by an individual organism, a tissue, or a cell, etc., at a given time and under certain conditions. Proteomics studies may also be directed at studying a specific subset of proteins of an organism (referred to as a subproteome), such as specific organelles (Fernández-Acero et al. 2010; Garrido et al. 2011; Kim et al. 2007). Concerning fungal biology, the most relevant and studied subproteome is the secretome, which is defined as the combination of native secreted proteins and the cellular machinery involved in their secretion. This subproteome consists primarily of cell wall degrading enzymes (CWDEs), many of which are known virulence factors in these organisms (Fernández-Acero et al. 2010). The importance of this information for unravelling pathogenesis and the infection cycle is undisputable.

16.1.1 Technical Constraints: From Past to Present

Proteins are the final effectors for most of the processes occurring in the cell and, currently, proteomics methodologies based on Mass Spectrometry (MS) offer new tools for gaining insights into these processes. MS is an analytical technique used to determine the molecular mass of a compound by measuring the mass/charge ratio (m/z) of ions under vacuum conditions. When it was first discovered, MS could not be applied to the study of proteins, but in the late 1990s, the development of "Matrix-Assisted Laser Desorption/Ionization" (MALDI) (Hillenkamp et al. 1991) and

"Electrospray ionization" (ESI) (Fenn et al. 1989), enabled mass spectrometry to be applied to the study of proteins and peptides. MALDI and ESI are two soft ionization techniques that allow gas phase ions to be obtained from large biomolecules without excessive fragmentation, and these techniques were crucial for the initial development of proteomics (Dass 2000).

Unlike the genome, which remains constant under all circumstances, the proteome is a highly dynamic system that varies depending on the environmental, physiological and/or pathological conditions of the organism. Proteins are regulated in their activity and level in response to internal or external events, either as a result of a differential expression of genes or depending on post-transcriptional events such as differential RNA splicing/editing and post-translational modifications (PTMs) of the synthesized peptides. More than 200 different types of PTMs have been described (Minguez et al. 2012). Among the best known are phosphorylation, glycosylation and proteolysis, which can redirect the biological function of any synthesized peptide. It can be said that the proteome of a given organism is the expression of its phenotype. Proteomics studies are among the most appropriate approaches for analyzing the biological processes that take place in an organism, as well as for understanding the complex network of interactions involved in cellular functions.

The approaches used in proteomics studies are labor-intensive because they depend heavily on the specific requirements of each particular study. Due to the dynamic nature of the proteome, each change in the assayed conditions entails a dramatic change in protein synthesis, PTMs, etc., and, therefore, in the results and conclusions of the study, analysis or experiment. In addition the proteome of phytopathogenic fungi undergoes continuous changes over time and it is crucial to decide the right time or stage when biological samples should be taken (mycelia, conidia, sclerotia, etc.) and to repeat the sampling at the same point in all replicates carried out. Therefore a good experimental design is crucial for obtaining good final results (Fernandez-Acero et al. 2007a). That design will include the choice of the best methodology for sample preparation, harvesting, protein extraction and separation, MS analysis, protein identification and quantification, as well as data analysis and interpretation. Concerning filamentous fungi, a key limiting factor is the choice of an effective protein extraction method capable of breaking open their exceptionally robust cell wall and of solubilizing all mycelial proteins free from contaminants. Polysaccharides, lipids, pigments and other fungal metabolites are among the contaminants that can reduce the protein extraction ratio and/or disturb the subsequent separation of the proteins. This is particularly important because it is impossible to study proteins if the extraction method is unable to extract them from their natural location in the cell. Briefly, a fungal protein extraction protocol should comprise at least three stages: homogenization, protein precipitation and cleaning, and the protocol should be adapted to the particular requirement of the species being studied. Advances in fungal protein extraction procedures have been reviewed by several authors (Garrido et al. 2011; González Fernández et al. 2010).

Once the proteomics researcher has decided on a good protein extraction method, a wide range of possibilities for protein separation and MS identification is available. Basically these possibilities can be classified into two categories: gel-based and gel-free methods. The standard proteomics study of an organism has tradition-

ally been based on the separation of the complex mix of proteins that make up its proteome by two-dimensional electrophoresis in polyacrylamide gel (2-DE), in combination with mass spectrometry identification. As we will see in detail in the next section, this is one of the most commonly used methods for proteomics research on *B. cinerea*. The 2-DE technique couples a first separation of proteins on the basis of their isoelectric point (first dimension) with a second separation according to their molecular mass (second dimension). The resulting gel is stained and digitalized. Once the resulting protein spot profile is analyzed and studied, proteins are picked directly from the gel and digested with a protease (typically trypsin), resulting in a set of peptides which are identified with a MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization type – Time of Flight) mass spectrometry by Peptide Mass Fingerprinting (PMF) or with a MALDI- TOF/TOF by Peptide Fragmentation Fingerprinting (PFF), and data base searching. 2-DE techniques allow large-scale separation with enough resolution power to resolve complex mixtures of proteins. The visual perspective of the proteome thus obtained allows the easy discrimination of PTMs and protein isoforms; these techniques do, however, present limitations in terms of (i) co-migrating proteins, and spots produced by abundant proteins that may mask the more scarce proteins (due to the large dynamic range of proteins present in a cell) thus complicating the analysis; (ii) inadequate sensitivity, depending on the staining used, which can also lead to difficulties in detecting low-abundance proteins; and (iii) low reproducibility among replicates.

Some of these limitations have been overcome with the emergence of gel-free technologies based on liquid chromatography (LC) methods, coupled directly to electrospray ionization mass spectrometers (LC-ESI-MS or LC-ESI-MS/MS). LC methods allow different separation principles such ion affinity, molecular mass, hydrophobicity, etc. to be combined in an orthogonal way to achieve the highest resolution such as, for instance, a combination of strong cation exchange (SCX) followed by a high-resolution reverse phase separation of peptides. That technology is known as “multidimensional protein identification technology” (MudPIT). This methodology provides several advantages over the traditional 2-DE approach; it makes possible “shotgun proteomics” studies, in which large-scale identification of complex protein mixtures may be carried out by MS/MS simultaneously and immediately after their chromatographic separation in a fast and reliable way and with high sensitivity. MudPIT techniques are particularly important for identifying low abundance proteins and others that are often difficult to resolve in gels because of their distinctive physical properties such as extreme hydrophobicity, high or low molecular weight, or high or low pI values. The methodology gives a wide coverage of proteins and therefore allows a wide range of proteomes to be described. It has also facilitated the development of different experimental approaches like, for instance, the combination of one-dimensional electrophoresis in polyacrylamide gel (1-D) and LC-MS/MS (1D-LC-MS/MS), to describe complex proteomes. This latter gel-based technique, with a previously-described 2-DE analysis, are the most widespread approaches currently used in *B. cinerea* proteomics research and is yielding good results in term of sensitivity and number of proteins identified.

At this point in time when, all in all, thousands of fungal proteins can be separated and identified relatively quickly and easily, the challenge in *Botrytis* proteomics

research is the application of new strategies aimed at improving the accurate quantitation of proteins. The methodology used to conduct a quantitative proteomics analysis is based on comparative analysis between the protein profiles obtained for the same organism, cell, etc., but under two different biological conditions, *e.g.* stimulated *vs*. unstimulated. Traditionally the mode of operation for carrying out a quantitative proteomics analysis is based on the analysis of the image obtained from 2-DE gels. In these studies proteins can be detected by staining them after 2-DE or by labeling them on lysine side chains before 2-DE with two different fluorescent cyanine dyes, each one having the same mass but different excitation and emission wavelengths. In the first approach, proteins from different samples are separated in different 2-DE gels, proteins are stained, the gels are digitalized and the obtained images are matched and compared with each other. In the second approach, however, proteins from different samples are fluorochrome-labeled, mixed and loaded onto the same 2-DE gel. The result is known as a "2-Dimensional fluorescence difference Gel Electrophoresis" (2-DIGE). After electrophoresis, the gel is digitalized using a fluorescence reader fitted with two different filters and two sets of images are obtained. Then images are overlaid and processed, revealing in a visual way the quantitative differences between the samples. Using this method, problems linked to reproducibility (local gel structure, running conditions, etc.) are avoided because the samples are run together. Since these approaches are gel-based they present the same limitations as mentioned above. The results rely on the size and intensity of the analyzed spots, and are highly dependent on the sensitivity and selectivity of the labeling-method used and the resolution of the processed image.

The progress made in recent years in LC-MS has provided a number of new, more reproducible and sensitive strategies for quantitative shotgun proteomics analyses based on the abundance or intensity of the analyzed peptides (MS) and/or fragments from these (MS/MS). These methods can be divided into two categories: labeled or label-free methods. In label-based approaches, proteins obtained from multiple biological samples under different conditions are tagged with stable-isotopic reagents whose physicochemical properties are identical except for their mass (one contains a light isotope and the other one contains a heavy isotope). Then samples are mixed and analyzed simultaneously (so reproducibility problems are eliminated) by LC-MS/MS using the isotopic-label as internal standard or references; this procedure gives accurate quantitative information based in peptide intensity. Label-based methods comprise different techniques which differ in their labeling chemistry and site of attachment, such as "Isotope-Coded Affinity Tag" (ICAT), "Isotope-Coded Protein Labeling" (ICPL), "Isobaric Tags for Relative and Absolute Quantification" (iTRAQ) and "Stable Isotope Labeling by Amino Acid in Cell Culture" (SILAC). Label-free approaches rely on the fact that the greater the abundance of a specific peptide the greater the number of spectral counts produced by that peptide; hence the number of spectral counts is linearly proportional to the concentration of the peptide measured. In a label-free approach peptides from different biological samples have to be compared across different LC-MS/MS runs, so results are less reproducible. To date (as we will see in next section) only label-free methods have been introduced, albeit gradually, into proteomics research on *B. cinerea.*

16.2 Proteomics Approaches to *B. cinerea*

16.2.1 Global Proteomics

Over the last 8 years an increasing number of original papers on *Botrytis cinerea* proteomics have been published; together these studies have reported a long list of proteins synthesized by this fungus in specific growth stages or assay conditions, and therefore provide valuable biological information, which will be discussed in the following sections. Most of these research reports deal with the study of the secretome, which has a particular relevance in phytopathogenic fungi, since it is considered a key element in the establishment and development of the infection cycle.

The initial proteomics study of *B. cinerea* was reported by Fernández-Acero et al. (2006). In this work, a protocol was optimized for protein extraction from *B. cinerea* mycelium using TCA-acetone protein precipitation, and the first proteome map of the fungus was described by 2-DE and MS analysis. Around 400 protein spots were resolved in a 2-DE Coomasie Blue Brillian (CBB) stained gel, covering the 5.4–7.7 pH and 14–85 kDa ranges. Twenty-two protein spots were identified by MALDI-TOF or ESI IT MS/MS, with a significant number of them related to virulence; these latter include Malate Dehydrogenase (MDH), Glyceraldehyde-3-Phosphate Dehydrogenase (GADPH) and cyclophilin. These findings, and others, showed the applicability of proteomics approaches as a starting point for the study of pathogenicity factors and for basic research on this plant pathogen in the post-genomic era. Following this initial paper, a second paper described an attempt to identify putative fungal virulence, and reported a comparative analysis carried out between two *B. cinerea* isolates (B.c 2100 and B05.10 strains) (Fernandez-Acero et al. 2007b) which differ in virulence and toxin production. It was carried out using the 2-DE MALDI TOF/TOF methodology. The resulting 2-DE protein profiles showed qualitative and quantitative differences between mycelial extracts from the two isolates, most of them associated with virulence factors. A total of 28 spots were identified with a significant number of isoforms of MDH, either over-expressed or exclusive to the more virulent strain (*B. cinerea* 2100). This protein plays a key role in the infection process, since it is involved in oxalic acid production (Lyon et al. 2004), pH decrease (Manteau et al. 2003), as well as in the biosynthesis and secretion of biotoxins (Durán-Patrón et al. 2004). In addition, four spots only present in *B. cinerea* 2100 were identified as a GADPH which, apart from its well-known role in the glycolytic cycle, has been reported to act as a virulence factor in several different organisms (Hernández et al. 2004; Alderete et al. 2001). These results lend support to the hypothesis that the different expression patterns revealed could be correlated with differences in virulence between strains, and again confirmed proteomics as an excellent tool for use as a first and direct step in the study of new fungal virulence factors in *B. cinerea*.

At that time, due to the absence of a protein and DNA database containing *B. cinerea* sequences, protein identification was achieved by MS *de novo* sequencing followed by MS BLAST alignment, yielding the identification against other species.

The emergence in 2007 of a data-base of *B. cinerea* proteins (http://www.broad.mit.edu) was a breakthrough, enabling researchers to apply a proteomics approach with a significant number of identified proteins. In the first study using this approach, the authors described the mycelial proteome of *B. cinerea* during cellulose degradation (Fernandez-Acero et al. 2009). Briefly, *B. cinerea* was grown in a minimal salt medium (MSM) supplemented with 1 % of carboxymethylcellulose (CMC) as a sole carbon source, in an attempt to simulate natural environmental conditions, based on the assumption that since cellulose is one of the major components of the plant cell wall, the use of CMC as a sole carbon source may reveal potential pathogenicity or virulence factors. Mycelial protein extracts were separated by 2-DE to obtain the proteome map. Two hundred and sixty-seven spots were selected for MALDI TOF/TOF MS analysis, resulting in 303 positive protein identifications, mostly representing non-annotated proteins. The authors classified these proteins into functional categories, showing the relevance of protein metabolism, modification processes, and oxidoreductase activity.

A breakthrough has recently taken place in the study of the *B. cinerea* mycelial proteome with the implementation of "shotgun" proteomics techniques. The adequacy of this methodology for mycelial *B. cinerea* protein identification and label-free quantification has been proven (Gonzalez-Fernandez et al. 2013). In this work the authors reported a comparative proteomics analysis of *B. cinerea* mycelium from two wild-type strains: B05.10 and T4 introducing label-free "shotgun" nUPLC−MSE methodology. In addition, they made the first comparative study between gel-based (2-DE) and gel-free/label-free (nUPLC-MS) approaches. A total of 225 (48 unique) and 170 (7 unique) protein species were identified by nUPLC−MSE in the B05.10 and T4 strains, respectively. Moreover, 129 protein were quantified in both strains. The authors concluded that the use of the label-free "shotgun" nUPLC−MS methodology to analyze the two *B. cinerea* wild-type strains isolated from different hosts allowed the quantification of differences in protein abundance. They obtained results complementary to the traditional gel-based approach (2-DE), which ultimately allowed the identification of strain-specific proteins. In short, the use of these two approaches provided comprehensive knowledge of the proteomes of *B. cinerea* strains.

Another interesting contribution concerning mycelial protein research has recently been made (Gonzalez-Fernandez et al. 2014). In this work, differences in the protein profiles of gel-based approaches among strains were used to carry out a comparative proteomics study of six wild-type strains. After image analysis, a total of 674 spots were considered, out of which 112 spots were strain-specific in this study. A total of 47 variable proteins were identified, some of them, such as malate dehydrogenase and peptidyl-prolyl cis–trans isomerase, had been reported as virulence factors. The main differences among strains were related to proteins involved in redox processes, such as mannitol dehydrogenase. These results again confirm the usefulness of proteomics approaches for gaining better understanding and insight into *Botrytis* phenotype variability.

16.2.2 **B. cinerea** ***secretome***

The first proteomics-based study of secreted proteins in *B. cinerea* was reported in 2009 by Shah et al. (2009a). Since then, an increasing number of secretome studies have been carried out, resulting in the identification of secreted proteins under different assay conditions related either to growth conditions, to developmental stages or to fungal strains. To date, five different approaches to the study the secretome of *B. cinerea* under different growth conditions have been published. Three of them deal with changes in the proteome/secretome of *B. cinerea* as a result of changes in carbon source. Shah et al. (2009a), used a gel-free LC-MS/MS approach to investigate the secretome produced by *B. cinerea* B05.10 on different hosts, under *in vitro* growth conditions. These were performed by inoculating *B. cinerea* on a nutritive agar medium containing either filtered pulp of ripe strawberry, red tomato or whole *Arabidopsis* plants and covered with a sterile cellophane membrane. Significant changes in the relative abundances and in the composition of the secreted enzymes, in a substrate-dependent manner, were observed; overall 89 *B. cinerea* proteins were identified from all the growth conditions. Out of this total seven proteins were observed to be common to all the samples, thus implying the constitutive nature of their synthesis and secretion. In respect of functional differences, these 89 proteins comprised transport proteins, proteins well-characterized for carbohydrate metabolism, peptidases, oxido-/reductases, and pathogenicity factors. These data provide important insights into how *B. cinerea* may use secreted proteins for plant infection and colonization.

In a second work, (Shah et al. 2009b) a "shotgun" proteomics approach was used to study the *B. cinerea* secretome on three different carbon sources in liquid cultures: highly-esterified pectin, partially-esterified pectin, and sucrose, as sole carbon sources, in order to demonstrate the impact of the degree of pectin esterification in the plant cell wall on fungal secretion, mimicking ripe and unripe fruits. The two pectin sources simulate fungal interactions with the expected host nutrient source; sucrose was used to define those enzymes considered constitutively produced. A total of 126 proteins secreted by *B. cinerea* were identified, 67 of which were observed in all three growth conditions. Thirteen *B. cinerea* proteins with functions related to pectin degradation were identified in the two pectin growth conditions, while only four were identified on sucrose as carbon-source. These results indicate that the secretion of most of the pectinases depends on the carbon substrate used by the fungus for growth. However no major differences were found in protein secretion when *B. cinerea* was grown with highly- *vs* partially-esterified pectin. It is therefore likely that the activation of *B. cinerea* from the dormant state to active infection is not dependent solely on changes in the degree of esterification of the pectin component of the plant cell wall. A similar study using a 2-DE MALDI TOF/TOF approach was carried out by Fernández-Acero et al. (2010) to determine the differences in *B. cinerea* protein secretion induced by five carbon sources and plant-based elicitors: glucose, CMC, starch, pectin and tomato cell walls (TCW). This work showed a different degree of complexity of the fungal response moving from a state of constitutive

fungal growth (by using glucose as a sole carbon source) towards more complex and possibly pathogenic secretory behaviour (induced by TCW). A total of 76 spots were identified yielding 95 positive hits that correspond to 56 unique proteins, including several known virulence factors (e.g. pectin methyl esterases, xylanases and proteases). These three proteomics studies are clear examples of how, by using modifications in the composition of culture media, the pathogenicity of *B. cinerea* can be induced or modified emulating *in vitro* conditions similar to those applicable *in planta*. The modification of culture media by plant compounds was used by Espino et al. (2010) to study the secretome proteins during the first 16 h, termed the "early secretome". By combining both approaches, 2-DE and LC-MS/MS, 116 proteins were identified, including several virulence factors. 2-DE was used to check the proteome profiles between the wild type and the mutant in an aspartic protease gene, and found that the specific spot disappeared from mutant gels.

Other interesting proteomics approaches to study the secretome from an environmental perspective have been reported. These works are aimed at studying the effects of metals stress and pH, respectively, on the secretome of *B. cinerea* using comparative proteomics based on 2-DE MALDI-TOF/TOF approaches. To define the effects of metals stress on the secretome of *B. cinerea*, four metals (copper, zinc, nickel and cadmium) were added to the culture media of the fungus (Cherrad et al. 2012) and a total of 116 protein spots were observed on 2-D gels; the findings indicate that the secretome signature seems to be metal-dependent. Fifty-five spots were associated with unique proteins, and functional classification revealed that the production of oxidoreductases and cell-wall degrading enzymes was modified in response to metals stress. These results clearly show that the fungus can adjust the production of secreted proteins in response to metals exposure. Finally the comparative analysis revealed that the accumulation of some secreted proteins increases specifically in response to one or to several metals. This proteomics study has thus identified a number of proteins that could be considered as potential biomarkers for monitoring pollution exposure.

Concerning the effect of ambient pH on the secretome of *B. cinerea*, Li et al. (2012) compared changes found in the secretome of *B. cinerea* grown in culture media buffered at pH 4 and 6. Forty-seven differential spots, corresponding to 21 unique proteins, were identified. Most of these proteins were cell wall degrading enzymes (CWDE) or proteases. At pH 4, more proteins related to proteolysis were induced, whereas most of the up-regulated proteins at pH 6 were CWDE. Analysis of gene expression using quantitative real-time PCR suggests that production of most of these proteins is regulated at the level of transcription. These findings indicate that *B. cinerea* can adjust its secretome profile in response to different ambient pH values.

All these observed changes in the profile of secreted proteins under different growth conditions (nutrient- or environment-dependent) are indicative of the versatility and adaptability of *B. cinerea* to different hosts and environmental conditions. These findings provide important information about the mechanisms employed by the fungus to access nutrients and to initiate its infectious cycle on a wide range of plant-hosts. Differences between the secretomes of six *B. cinerea* strains were

analyzed by 1-DE coupled to LC-MS/MS (Gonzalez-Fernandez et al. 2014), and 51 variable proteins were identified, including endopolygalacturonases, aspartic proteases and the cerato-platanin protein. All of them have been reported before as virulence factors.

Even though plant components can usefully be incorporated in the cultures, the real situation can only be studied during actual plant-*Botrytis* interactions. However, in such studies, fungal proteins seem to disappear, masked by the huge amount of plant proteins (Mulema et al. 2011) or they are reduced to only a few spots (Cilindre et al. 2008). A deep analysis of tomato infected by *B. cinerea* was tackled by Shah et al. (2012). By using 1-DE plus LC-MS/MS, 79 *B. cinerea* proteins were found during the infection of three different tomato-ripening stages. This study allowed the identification of 15 common proteins from the fungus, including CWDE and other virulence factors.

16.3 Proteomics – A Long Future Ahead in the Biology of *Botrytis*

Proteomics-based approaches to the study of *B. cinerea* have shown, among others, the potent capacity of this science to identify accurately the many protein candidates involved in specific biochemical pathways or phytopathological events. In order to obtain a global perspective, all available *B. cinerea* protein identifications performed in the various different proteomics approaches were summarized and analyzed together. During this "experiment", we found several problems. Despite difficulties in comparing data published in different journals under different formats and those published in the pre- and post-genomic era (Fernandez-Acero et al. 2006; Amselem et al. 2011; Staats and Van Kan 2012), a file containing 682 non-redundant sequences was obtained from the 12 papers available. Taken together, proteomics research exploring the *Botrytis cinerea* proteome covered only a very small proportion (4.4–6.6 %) of the fungal genome, depending on the genome sequence and its annotation (gene prediction). This could mean that we are only at the beginning of a long distance race. There are several different explanations for this poor coverage. First there are, to date, only a few studies published, most of them based on 2-DE, in which, in the best cases, only hundreds of proteins are resolved. Nowadays, LC-based studies are identifying increasingly larger numbers of proteins. Another reason may be associated with the experimental approach used. Most of the published data has been obtained using general culture media; and some of them include plant components to induce specific fungal responses. Only a few studies have applied proteomics approaches directly to plant-*Botrytis* interactions, where fungal proteins only represent a small percentage compared to those of the plant components. Future proteomics approaches will increase this ratio by studying specific subproteomes, varying the components of the media to induce new sets of proteins or, finally, with wide application of gel-free proteomics technologies to increase the

number of identified proteins. Recently, targeted proteomics was named the technology of the year by an important journal (Marx 2013). This approach will allow the search for specific proteins from a complex mixture, making it possible to find weakly represented proteins.

In order to measure the relevance of the identification within the overall biology of *Botrytis*, and check which are the principal groups of proteins that had been found, all identified sequences were categorized using the gene ontology classification (AgBase; http://www.agbase.msstate.edu/). **Cellular component** classification includes those proteins which are part of something larger, such as anatomic structures, organelles, etc. The first category found for the identified proteins corresponds to mycelial proteins such as intracellular, cell or cytosolic proteins, which made up around 36 % of all the identified proteins. This is quite a surprising result since most of the identifications were extracted from secretome publications. Proteins from extracellular regions appeared as the fifth category. However, when the whole content of the database was uploaded, extracellular regions accounted for less than 1 %. In previous studies (Fernández-Acero et al. 2010) it has been established that the protein sequences of the *B. cinerea* database that present secretion signals (Signal P) comprise around 15 % of the total, showing that these secreted proteins are included in other categories (e.g. endoplasmatic reticulum, protein complexes, etc.). This finding must be associated with the close relationship that secreted proteins have with the membranes during secretion processes.

Protein categorization by **biological processes** shows those protein involved in a series of events that take place in one or more distinct steps (Fig. 16.1). Major categories represented in identified proteins are concerned with metabolism (biosynthesis, catabolism, small molecule and carbohydrate metabolic processes) while the total genome presents major categories concerned with other biological processes (nitrogen, small molecules or transport metabolic processes). This is probably due to the use of synthetic culture media, which are normally artificially enriched. Other differences show that identified proteins present over-represented categories such as transport and cell cycle, cellular protein modification or DNA metabolic processes. The **molecular functions** of identified proteins (Fig. 16.2) describes the different catalytic or binding activities of the proteins that occur at a molecular level. A comparison of all the categories obtained between identified proteins and the whole genome makes it clear that most of the categories of identified proteins are still under-represented. Oxydoreductase activity percentages are similar but activities such as kinases or transmembrane transporters, crucial for the signal transduction pathways, are an average of 7.5 times less represented in the identified proteins than in the database.

The observed differences between the proteomics dataset and all predicted proteins may have several explanations. Mainly, most of the synthetic culture media used contain similar compounds, and these probably inhibit the synthesis of whole peptides that may be useful for the fungus during its necrotroph/hemi-biotroph/biotroph lifestyle (Tudzynski and Kokkelink 2009). Moreover, at present, there are only a few studies focused on the identification of fungal subproteomes, mainly the secretome. The *B. cinerea* proteomics community must develop new strategies for

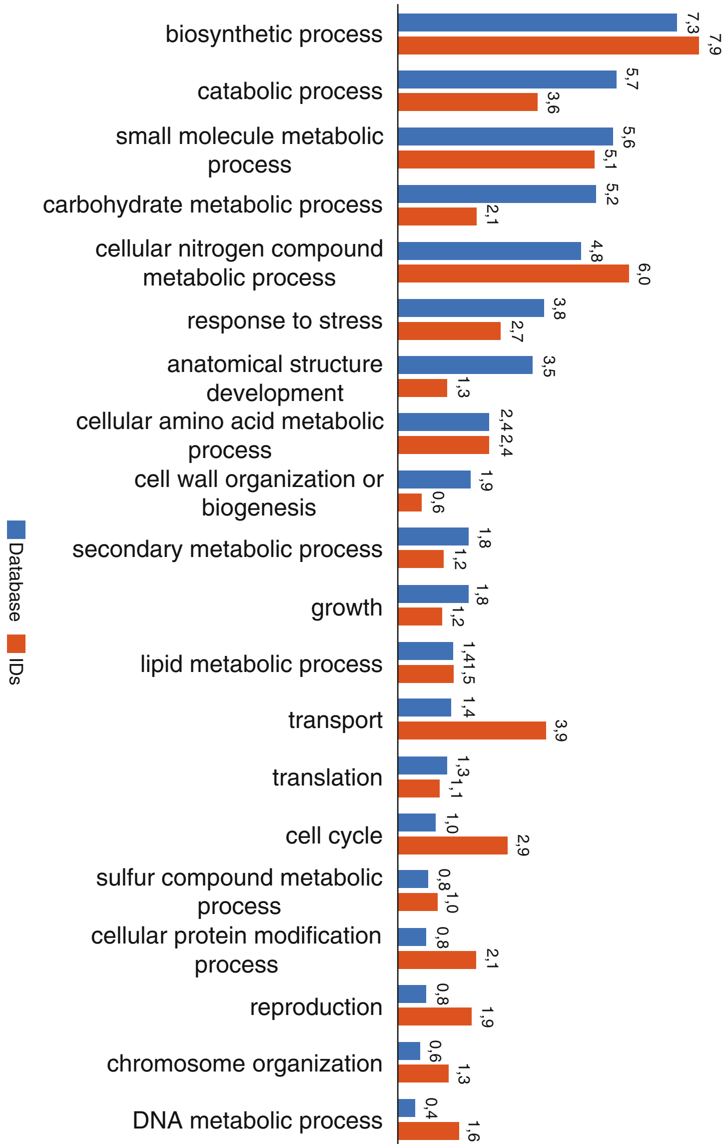

Fig. 16.1 Gene ontology classification by biological process. Percentages of *B. cinerea* proteins listed in the genome database vs. proteins from proteome datasets

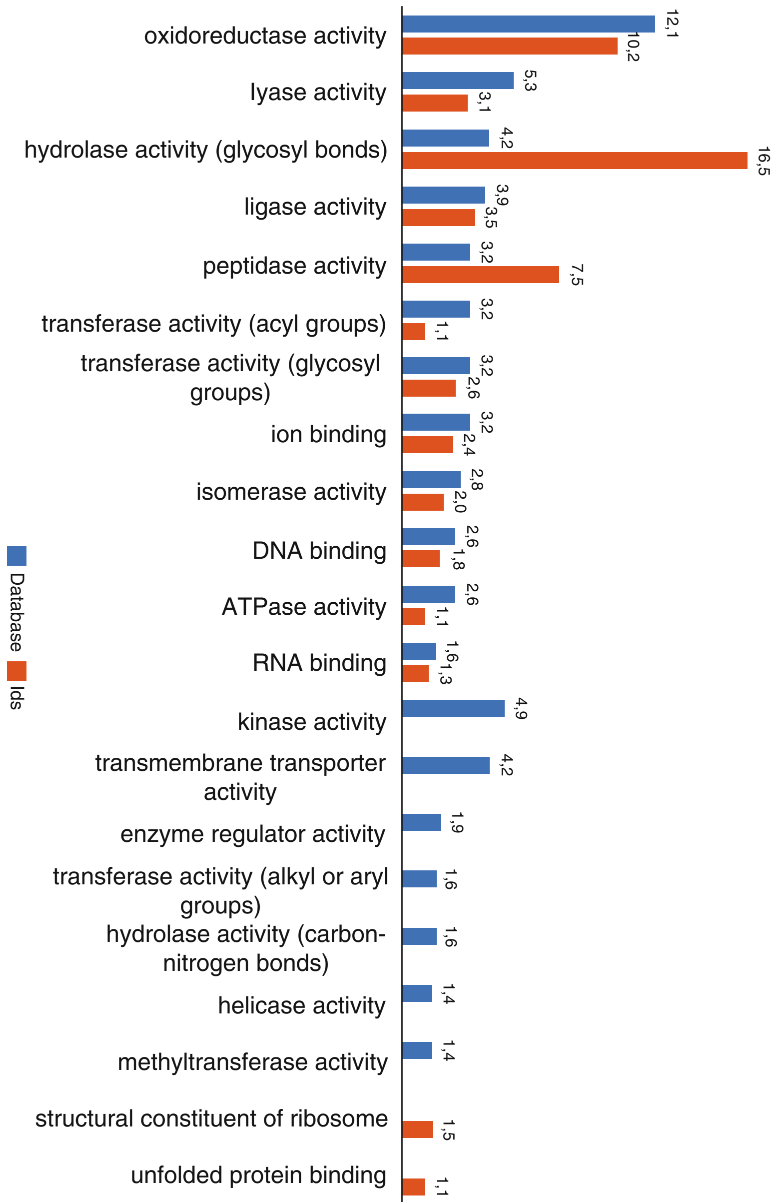

Fig. 16.2 Gene ontology classification by molecular function. Percentages of *B. cinerea* proteins listed in the genome database vs. proteins from proteome datasets

inducing the synthesis of new proteins by varying the media content, emulating natural conditions to collect under-represented proteins. New approaches to fungal subproteomes must be developed as well, allowing the study of those proteins specifically produced during discrete stages of the life cycle (conidia, appressoria, sclerotia, etc.). Such studies are now running in several laboratories. During the next few years, the proportion of genome coverage will probably increase accordingly.

16.4 Biological Relevance of Proteomics Approaches

As described above, proteomics approaches are making it possible to detect efficiently global protein production under specific conditions. They allow the detection of proteins involved in specific biological events. Moreover, in other biological systems where transcriptomics have been compared with proteomics analyses, it has been stated that the proteome is the "relevant" set for analysis (Rossignol et al. 2009). This conclusion is based on significant differences that had been described previously (Haider and Pal 2013; Hack 2004) between transcriptomics and proteomics data. This comparison showed a correlation between both datasets in the range from 0.46 to 0.76; thus sometimes, but not always, variations in protein abundances can be predicted from the transcriptome. Currently, there is no global analysis of this correlation in *B. cinerea*. However, one thing seems to be clear: if, after several controls, replicates and statistical analyses, one protein is found to be present in a specific context, it must be significant, independent of whether we are able to detect its mRNA, as has been widely discussed. This is one of the most important impacts that proteomics has had on our series of biological dogmas.

However, the main aim of the proteomics research activity in *B. cinerea* has been directed to the quest for those peptides/proteins involved in the different steps of the pathogenicity process. This aim has been present from the first papers (Fernandez-Acero et al. 2007b) showing that proteomics approaches were capable of detecting several candidates as virulence factors without previous exclusion criteria. It allows the recovery of dozens or hundreds of candidates from a single experiment. However, the downside to establishing a direct relationship between identified proteins and virulence or pathogenicity factors is the great amount of work that must subsequently be done in order to discriminate real virulence factors from other proteins. Normally, this discrimination is done by constructing knockout mutants of each candidate and evaluating the influence during plant infection. Due to the power of proteomics technologies to identify hundreds of potential candidates, the functional validation of the candidate genes to elucidate their specific roles in the pathogenicity process will rapidly become the bottleneck.

An example of this objective is the role of two secretome components. Aspartic proteases are the main components of the *B. cinerea* secretome (Fernández-Acero et al. 2010). A deep molecular analysis of these components revealed that deficient mutants show the same level of virulence (ten Have et al. 2010). However their usefulness in removing haze-forming proteins during wine fermentation has been

highlighted (Van Sluyter et al. 2013). In another study, Espino et al. (2010) found a BcSpl1, a cerato-platanin, in the fungal secretome. Further molecular analysis has shown its phytotoxic activity and its role as a virulence factor (Frías et al. 2011). In spite of these advances, there are still hundreds of identified proteins waiting for molecular analysis, representing a source of potentially valuable biological information. Finally, the capacity of proteomics approaches to find virulence factors is indisputable, since new virulence factors usually appear during proteomics analyses.

16.4.1 Possible Applications of Proteomics in **Botrytis**

Another significant aspect for review is the capacity of proteomics approaches for revealing new therapeutic targets for fungicide design. At the moment, however, this potential is only a possibility. The basis of this idea is that most of the drug targets are proteins. The development of fungicides based on molecular information may open possibilities for the production of environmentally-friendly drugs, with low impact, and with high levels of species-specificity. The reasons why there is a lack of molecular-based fungicides are still unclear, even though the necessary scientific strategies and technologies seem now to exist. New proteomics-based technologies have shown their capacity to serve as effective tools for fungicide design (Fernández-Acero et al. 2011) given the discovery of new disease factors that may be candidates for new therapeutic targets. Tools such as peptide aptamers and RNA silencing may be future instruments for crop protection. In this context, the potential use of modified natural compounds to inhibit specific fungal activities has been revealed as a possibility. This approach has been termed "chemoproteomics", defined as the use of biological information to guide chemistry (Beroza et al. 2002), which is presented as a new methodology to accelerate drug discovery in the post-genomic era. In spite of this optimistic scenario, there are various serious problems in transferring these technologies to crop management; these include, for example, maintaining activity in plants, metabolite stability, and obviously the costs of development and production. None of these technologies has yet led to a recognizable fungicide development candidate (Tietjen and Schreier 2013).

16.5 Future Perspectives

Nowadays, the term "post-genomic era" is widely used to refer to the present stage of the molecular sciences. This term is mainly used to refer to advances in transcriptomics and high throughput mutagenesis, but "the name of the game is proteomics" (Brower 2001). However, for the community studying *B. cinerea*, it is still an open field where only a few milestones have been reached revealing the potential

importance of proteomics; therefore many new problems, specifically those linked to fungal biology, remain to be solved.

To this end, the publication of the *B. cinerea* genome databases, after the publication of the genome sequences (Amselem et al. 2011; Staats and Van Kan 2013), has been crucial. This has enabled proteomics researchers to access directly the molecular information, making it possible for them to identify *B. cinerea* proteins directly, without the need to search for homologous proteins in other fungi or organisms. However, the slowness in developing specific bioinformatic tools is still a weakness, as these tools may help to understand the large set of proteins identified, avoiding the loss of relevant biological information. For example, specific fungal gene ontology queries and analyses of protein-protein interactions and differential pathways may improve the number of conclusions obtained from each approach. Moreover, the development of specific software to predict the role of a protein as a fungicide target may help to usher in a new era of directed fungicides. Most of these tools are a reality in other biological systems (Chen et al. 2007; Huan et al. 2007), suggesting that their use in *Botrytis* studies will soon become feasible.

Most of the proteomics approaches described so far with *Botrytis cinerea* only cover mycelia and/or the secretome. However the fungal life cycle includes many fungal stages, anatomical structures, organelles, etc. whose proteomes remain to be elucidated. The analysis of new subproteomes will help to find interesting new proteins that are needed to understand better the complexity of fungal biology. In this context, there are two other proteomics approaches that seem to be missing in *B. cinerea* – first, the analysis of post-translational modifications (PTMs) of fungal proteins and second, quantitative proteomics approaches. Around 200 different PTMs have been described. These modifications are the bridge between extracellular and intracellular environments, and are crucial for signal transduction cascades that direct fungal behavior and virulence, but only one paper has been published recently, on the phosphoproteome of *B. cinerea* (Davanture et al. 2014). As mentioned previously, the most recently developed proteomics technologies are also known as "quantitative proteomics"; this is related to the specific labeling of proteins with specific isobaric tags. As far as we know, this methodology has not yet been used for *B. cinerea*. It would allow the specific detection and quantification of differential proteins, thus revealing their role under assay conditions. We can assume that more subproteomes, PTMs and quantitative studies will be available soon. Another set of interesting proteins still unresolved are those involved directly in the plant pathogen interaction. These approaches represent a more realistic way of studying the fungal weapons deployed for invading plant cells and defeating the plant defense mechanisms. Some studies have been undertaken, but the fungal "hits" obtained are either few in number or non- existent. Greater success in the identification of fungal proteins may be obtained by selective extraction enrichment and/or labeling.

One of the most promising proteomics approaches to *B. cinerea* is "targeted proteomics". This set of strategies was selected as the method of the year 2013 by the journal Nature Methods (Marx 2013). In brief, it will enable the study of specific proteins by MS/MS from a whole set, allowing quantitative assays to specifically

answer hypothesis-driven questions. The detection of specific peptide ions is made possible by focusing the MS instrument (typically, a triple quadrupole) for a preselected set of peptides. It will allow the specific detection of proteins in a wide set of samples, with a higher level of sensitivity. Sequential window acquisition of all theoretical fragment-ion spectra (SWATH), with improvement by independent data acquisition, will change the perspective of proteomics studies from one based on a large set of identified proteins to a smaller set of more relevant identifications. All these improvements should become publicly available within the next few years. They will transform proteomics technology into an indispensable and commonly-used tool for understanding the biology and virulence of *B. cinerea.*

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